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Host RNA polymerase inhibitors encoded by ϕKMV-like phages of *pseudomonas*

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ABSTRACT

Escherichia coli bacteriophage T7 is a founding member of a large clade of podoviruses encoding a single-subunit RNA polymerase (RNAP). Phages of the family rely on host RNAP for transcription of early viral genes; viral RNAP transcribes non-early viral genes. T7 and its close relatives encode an inhibitor of host RNAP, the gp2 protein. Gp2 is essential for phage development and ensures that host RNAP does not interfere with viral RNAP transcription at late stages of infection. Here, we identify host RNAP inhibitors encoded by a subset of T7 clade phages related to ϕKMV phage of *Pseudomonas aeruginosa*. We demonstrate that these proteins are functionally identical to T7 gp2 *in vivo* and *in vitro*. The ability of some *Pseudomonas* phage gp2-like proteins to inhibit RNAP is modulated by N-terminal domains, which are absent from the T7 phage homolog. This finding indicates that *Pseudomonas* phages may use external or internal cues to initiate inhibition of host RNAP transcription and that gp2-like proteins from these phages may be receptors of these cues.

Introduction

During the infection of *Escherichia coli* by bacteriophage T7, only ~2% of phage genome corresponding to genetically defined left end enters the infected cell immediately after the phage attachment (Garcia and Molineux, 1995; Kemp et al., 2005). The remaining DNA is brought in by transcription, first by *E. coli* and then by T7 RNAP (Kemp et al., 2004). The leading part of the genome contains strong early promoters recognized by the host σ^{70} RNAP holoenzyme ($E\sigma^{70}$). Transcription by $E\sigma^{70}$ from the early promoters causes at least 7 kbp of the ~40 kbp T7 genome to enter the bacterial cell (Kemp et al., 2004). The mRNAs produced from these promoters code for several phage proteins involved in the shut-off of host defenses, such as gp0.3, an antirestriction protein (Studier, 1975; Mark and Studier, 1981; Walkinshaw et al., 2002), and gp0.7, a protein kinase that phosphorylates the β' subunit of host RNAP and affects its termination properties (Severinova and Severinov, 2006). *E. coli* RNAP also transcribes gene 1, which encodes a single-subunit, rifampicin-resistant viral RNAP. The latter enzyme normally completes the genome internalization process by transcription from its own promoters, which are located throughout the genome (Garcia and Molineux, 1999).

T7 RNAP transcribes the middle and late genes of the phage (Fig. 1). Genes from the former group mostly code for proteins involved in phage DNA replication. Genes from the latter group encode structural proteins of the T7 virion. The product of phage middle gene 2 is a potent inhibitor of host RNAP (Hesselbach and Nakada, 1977). Gp2 is a 7 kDa protein that binds to the host RNAP β' subunit downstream jaw domain and prevents open promoter complex formation by $E\sigma^{70}$ (Nechaev and Severinov, 1999). Gp2 has no effect on transcription by T7 RNAP (LeClerc and Richardson, 1979).

T7 gene 2 is an essential gene. Analysis of infection of non-permissive cells by T7 gene 2 amber ($T7^{2am}$) mutants reveals that the infection is blocked at the stage of packaging of concatemeric viral DNA into virion heads (LeClerc and Richardson, 1979). The absence of gp2 can be complemented by treating $T7^{2am}$–infected cells with rifampicin, an inhibitor of host RNAP, after the onset of middle gene transcription (Ontell and Nakada, 1980; Mooney et al., 1980). The result thus suggests that the only essential function of gp2 is the inhibition of host RNAP transcription, which apparently becomes deleterious late in infection. The exact mechanism by which host RNAP interferes with phage development is not known,

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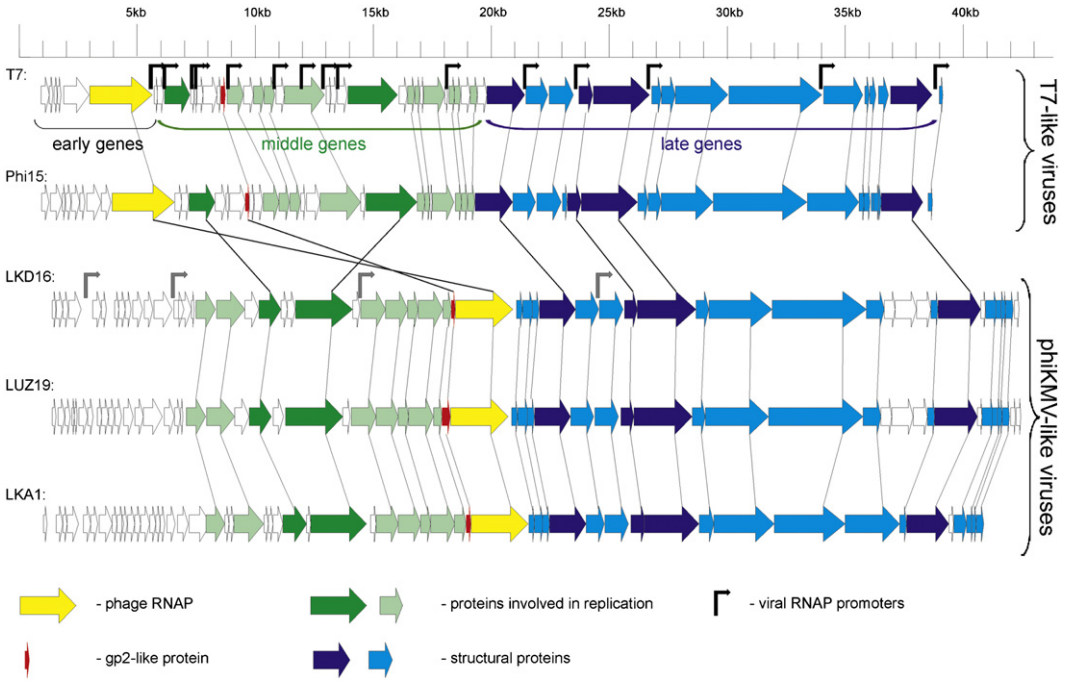

Fig. 1. Comparison of T7- and ϕKMV-subgroup phage genomes.The genome of T7 phage is schematically presented at the top and aligned with a phi15 (a T7-subgroup *Pseudomonas* phage) and ϕKMV-subgroup *Pseudomonas* phages LKD16, LUZ19, and LKA1. In T7, genes belonging to different temporal expression classes (early, middle, and late) are indicated. Some replication (dark green) and structural (dark blue) genes are homologous in all five genomes, as are the viral RNAP genes (yellow). Host RNAP inhibitor genes are shown in red. Genes indicated by lighter shaded of green or blue are conserved within T7- of ϕKMV-subgroups only. Rightward arrows indicate viral RNAP promoters, known (for T7) or predicted[18] (for LKD16).

however, disruption of one of the early promoters, A3, makes gp2 dispensable (Qimron et al., 2008; Savalia et al., 2010). It has been proposed that when the slower moving host RNAP transcribes into extended middle and late gene clusters of the phage, it becomes an obstacle to the much faster viral RNAP, causing the latter to stall and recruit phage DNA packaging/concatemer cleavage machinery at aberrant sites, leading to generation of less-than-unit length non-functional phage genomes (Qimron et al., 2008).

T7 is a founding member of a very large clade of phages of the *Podoviridae* family (Molineux, 2006). Many members of the family (sometimes referred to as "the T7 supergroup" (Hardies et al., 2003) and now formally classified as the *Autographivirinae* subfamily within the ICTV IXth report) encode a single-subunit viral RNAP. While some phages, belonging to the T7-subgroup, are very similar to T7 in their genomic organization and likely share a common strategy of infection, others are quite different. In particular, a clearly distinct subgroup, named after the *Pseudomonas aeruginosa* ϕKMV phage (Lavigne et al., 2003; Ceyssens et al., 2006) was described. Phages from this group infect bacteria of the *Pseudomonas* genus and differ from T7 and its close relatives in several respects, most obviously in the position of the viral RNAP gene. In ϕKMV-like phages, the viral RNAP gene is located close to the middle of the genome, between the replication and structural gene clusters (Fig. 1). Therefore, the process of phage DNA injection in host cell, and the temporal control of gene expression of ϕKMV-like phages must differ significantly from those operating in the T7 subgroup. In fact, no homologs of T7 gene 2 are listed in available annotations of ϕKMV-related phage genomes (Lavigne et al., 2003; Ceyssens et al., 2006; Kulakov et al., 2009). However, since ϕKMV-like phages probably rely on viral RNAP transcription for expression of at least their structural genes, one can hypothesize that a host RNAP inhibitor(s) either analogous or homologous to gp2 proteins encoded by T7 subgroup phages may be also encoded by ϕKMV-related phages. In this paper we identify host RNAP inhibitors encoded by ϕKMV-like phages and show that these proteins share a common RNAP binding site with T7 gp2 and can functionally substitute for T7 gp2 during the infection. We propose that most, or, perhaps, all phages encoding their own RNAP, also encode inhibitors of host RNAP that ensure orchestrated transcription of phage genes during the infection.

Results

Identification of phage proteins interacting with pseudomonas RNAP in cells infected with ϕKMV-like phages

Co-affinity purification coupled with mass spectrometric identification is a powerful method to identify unknown phage proteins binding to host RNAP (Savalia et al., 2008; Westblade et al., 2008). To apply this approach to ϕKMV-like phages, we constructed a *P. aeruginosa rpoC::PrA* strain encoding a Protein A tag attached to the C-terminal end of the RNAP β' subunit. The strain was viable and indistinguishable from parental wild-type strain at laboratory conditions. The strain was infected with ϕKMV-like phages LKA1 or LUZ19 (Ceyssens et al., 2006), cells were harvested 10 min post-infection and processed for affinity purification of RNAP. Mass-spectrometric identification revealed the presence of Eσ^{70} components (RNAP α, β, β', ω, and σ^{70}) and host transcription factor NusA. In addition peptides from phage LKA1 protein gp36 and LUZ 19 gp25.1 were also detected (Supplementary Tables 1 and 2), indicating that these proteins might interact with host RNAP in infected cells.

Bioinformatic prediction of ϕKMV-like phage proteins similar to T7 gp2

Phage LKA1 protein gp36 and phage LUZ19 gp25.1 are small proteins that, according to available annotations, have no similarity to proteins of known function in public databases. C-terminal parts of both proteins are similar to each other and both proteins are encoded by genes located immediately

upstream of respective viral RNAP genes (Fig. 1). Similar proteins are encoded by other known ϕKMV-like phages (Fig. 1). While a PSI-BLAST search of NCBI NR database with default parameters using T7 gp2 sequence (**GI: 9627441**) as a query does not retrieve any similar sequences from phages of the ϕKMV group (Ceyssens et al., 2006), more careful analysis presented below predicts that LKA1 gp36 and LUZ19 gp25.1 are related to T7 gp2. When gp2 homolog from T7-like *Pseudomonas* phage phi15, (**GI: 326424972**) is used as a query and the search is performed only for database subset representing sequences from *Caudovirales* with the PSI-BLAST parameters modified for small proteins (see details in Material and Methods), a sequence of ϕKMV-like *Pseudomonas* phage LKD16 gp25b (**GI: 158345052**) is retrieved with statistically significant E-value of 9×10^{-5}. A search started from the latter sequence identifies homologs in all available genomes of phages from the ϕKMV subgroup infecting *P. aeruginosa*. Identified proteins include LKA1 gp36 and LUZ19 gp25.1 that, based on co-purification data (above), interact with host RNAP. Initial analysis failed to identify a protein similar to T7 gp2 encoded by ϕKMV, the prototypical member of the phage group analyzed here. However, more careful analysis revealed that published annotation (Lavigne et al., 2003) has overlooked a ϕKMV ORF between nucleotide positions 17880–18024 **(AJ505558**) that codes for a protein identical to LKD16 gp25b.

We selected non-identical sequences detected by PSI-BLAST searches to build a multiple alignment using MUSCLE program (see Supplementary Figure 1). An alignment of T7 gp2 with a subset of predicted homologs from phi15 gp16, LKD16 gp25b (identical to gp28 encoded by phage PT5, **GI: 195546669** and the ϕKMV protein missed in the original annotation), LUZ19 gp25.1 (**GI: 167600471**, identical to phiKF77 gp29, **GI: 225626352**), and LKA1 gp36 (**GI: 158345170**) is presented in Fig. 2. As can be seen, a putative homolog from LUZ19 (gp25.1) is considerably longer than other proteins, with a sequence similar to T7 gp2 located at its C-terminal end. The ~60 aminoacid N-terminal extension of gp25.1 has no similarity to protein sequences in public databases.

Gp2-like proteins encoded by pseudomonas phages complement the growth of T7 gene 2 amber mutant

T7 with an amber mutation in gene 2 does not form plaques on lawns of non-suppressing MG1655 *E. coli* cells due to strongly decreased burst size (LeClerc and Richardson, 1979). The mutant phage plates normally on a suppressing strain IJ511 (Table 1). When the host contains a wild-type copy of gene 2 on a pET expression plasmid, plaques of normal size form (Cámara et al., 2010; Table 1). The plasmid-borne gene 2 is transcribed from a promoter recognized by T7 RNAP. The plasmid-borne gene is thus not transcribed in uninfected host, however, during the infection, transcription of plasmid-borne gene 2 by virus-encoded RNAP occurs, followed by complementation of growth defect. Genes coding for predicted gp2-like proteins from *Pseudomonas* phages were cloned into pET plasmid vectors, plasmids were introduced into non-suppressing *E. coli* cells, and the ability of T7 carrying an amber mutation in gene 2 to form plaques on lawns formed by these cells was determined. The results, presented in Table 1, demonstrate that cells carrying plasmid-borne genes from phi15, LKD16, and LUZ19, but not from LKA1, complemented the growth defect of the mutant phage. The result thus suggests that the products of phi15, LKD16, and LUZ19 genes may act as inhibitors of *E. coli* RNAP. The result with the phi15 product is an expected one, since this protein is very similar to the gp2 homolog encoded by *Pseudomonas* phage gh1 (Kovalyova and Kropinski, 2003), which we previously showed to inhibit transcription by *E. coli* and *P. aeruginosa* RNAP *in vitro* through the same mechanism as T7 gp2 (Nechaev et al., 2003). The result with LKD16 and LUZ19 products suggests that phages of the ϕKMV subgroup also encode a host RNAP inhibitor. This conclusion, however, appears to be incompatible with the fact that the LKA1 protein gp36, which is clearly related to LUZ19 and LKD16 proteins, does not complement T7 2^{am} growth. Inspection of the LKA1 gp36 sequence (Fig. 2) reveals the presence of a short N-terminal extension that is absent in other proteins. We hypothesized that this extension may affect the ability of the C-terminal part of the protein to inhibit *E. coli* RNAP. Accordingly, pET plasmid expressing gp36 lacking the N-terminal extension (gp36-short, starting with methionine corresponding to Leu^{16} of the full-length protein) was created and tested for complementation. Partial complementation of T7 2^{am} defect was observed on cell lawns expressing the shortened version of the protein (plaques of <1 mm diameter

Table 1
Complementation of T7 2^{am} growth defect by overproduction of plasmid-encoded T7 gp2 or similar *Pseudomonas* phage proteins.

Host	E.O.P.	Size of plaques
IJ511 (Su)	1	+++
MG1655 (wt)	0	–
MG1655 + pET33-gp2 (T7)	−1	+++
MG1655 + pET19-gp16 (phi15)	−1	+++
MG1655 + pET19-gp25 (LKD16)	−1	+++
MG1655 + pET19-gp25.1 (LUZ19)	−1	+++
MG1655 + pET19-gp25.1 short (LUZ19)	−1	+++
MG1655 + pET19-gp25.1 SC (LUZ19)	0	–
MG1655 + pET19-gp36 (LKA1)	0	–
MG1655 + pET19-gp36short (LKA1)	−0.5	+
MG1655 + pET19-gp36 C4A (LKA1)	−0.5	+
MG1655 + pET19-gp36 C7A (LKA1)	−0.5	+
MG1655 + pET19-gp36 C4A C7A (LKA1)	−0.5	+

~200 plaque forming units of T7 2^{am} were plated on lawns of *E. coli* strains with or without indicated plasmids (column 1) and after overnight growth efficiency of plating (column 2, defined as the ratio of plaques formed on indicated cell lawns to the number of plaques formed on suppressing strain IJ511) and the size of phage plaques (column 3) was recorded. "−" indicates no visible plaques present; "+" indicates plaques less than 1 mm in diameter, while "+++" indicates plaques, with diameter of more than 3 mm.

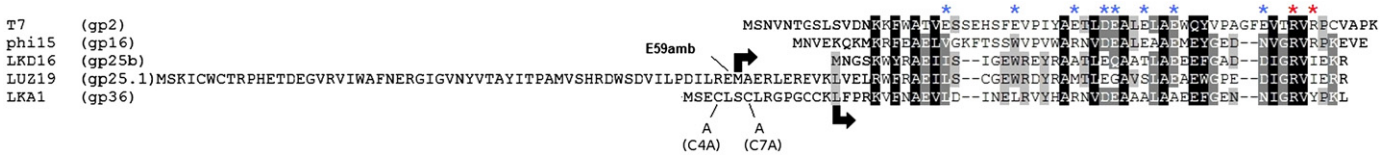

Fig. 2. Multiple sequence alignment of T7 gp2 with putative homologs from *Pseudomonas* phages. The T7 gp2 sequence presented at the top (single aminoacid code) is aligned with similar sequences from indicated *Pseudomonas* phages. The alignment was prepared using the GENEDOC software. Intensity of the background corresponds to degree of conservation; hyphens indicate gaps. For proteins from LUZ19 and LKA1, arrows indicate the positions from which shorter versions of the proteins began. The position of the amber codon introduced in the long version of gp25.1, as well as the sites of aminoacid substitutions of gp36 N-terminal cysteines are indicated (see text for details). Red and blue asterisks above the T7 gp2 sequence indicate T7 gp2 residues important for RNAP binding and inhibition, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared to >3 mm diameter plaques formed on cells expressing T7 gp2).

The N-terminal extension of gp36 contains four cysteine residues that may bind a metal ion, potnetially forming a Zn-finger-like structure. Plasmids expressing gp36 versions with substitutions of cysteine residues for alanines (C4A, C7A, and a double mutant C4A C7A) were created and tested for complementation. Cells harboring the mutant gp36 expression plasmids complemented the growth defect of T7 2^{am} phage to the same extent as cells expressing a truncated version of the protein (Table 1). We therefore conclude that the N-terminal extension of bacteriophage LKA1 gp36 protein negatively modulates its ability to inhibit *E. coli* RNAP. We speculate that this modulation may be dependent on gp36 ability to chelate metal ions.

If its N-terminal extension affects the ability of gp36 to inhibit bacterial RNAP, then one needs to regard complementation data with LUZ19 gp25.1, a much longer protein, with caution, since it is possible that complementation observed is due to a shorter protein synthesized from an internal translation initiation site. Indeed, cells harboring a plasmid expressing the C-terminal part of gp25.1 ("gp25.1 short", amino acids 60–121) fully complemented the growth of T7 2^{am} phage (Table 1). To determine whether a short version of gp25.1 is indeed responsible for complementation of mutant T7 growth in cells harboring a plasmid expressing full-length gp25.1, an expression plasmid with LUZ19 gene *25.1* containing an amber codon at position 59 was created. The stop codon was expected to prevent the synthesis of the longer version of gp25.1 but should have had no effect on the short version production if such version existed. As can be seen from Table 1, cells harboring such a plasmid did not support the growth of T7 2^{am} phage. The result thus proves that the longer version of gp25.1 is solely responsible for complementation observed. This conclusion agrees with MS/MS identification that revealed a peptide corresponding to LUZ19 gp25.1 residues 46–57 in affinity purified RNAP from infected *P. aeruginosa* cells (Supplementary Table 3). This peptide is present in full-sized gp25.1 but absent from the shorter version. Overall, analysis of gp25.1 indicates that N-terminal extensions as such have no effect on T7 gp2 homologs ability to inhibit *E. coli* RNAP, lending further support to a notion that a specific structure adopted by the four-cysteine containing N-terminal extension of gp36 is responsible for its apparent inactivity in the complementation assay.

E. coli cells JE1134 harbor a deletion of RNAP β' residues 1149–1190 (Ederth et al., 2002). The deletion destroys the T7 gp2 binding site, the β' downstream jaw, and thus mimics the absence of gp2 (Savalia et al., 2010). As a result JE1134 cells are non-permissive for wild-type T7. Likewise, *E. coli* 7009 cells, harboring a charge altering E1188K substitution in the β' subunit, are also non-permissive to gp2, since the mutation prevents the interaction of gp2 with its target (Nechaev and Severinov, 1999). JE1134 or 7009 cells harboring T7 gp2 overproduction plasmid did not complement the growth of $T7^+$, as expected. Likewise, wild-type T7 was unable to form plaques on lawns of JE1134 or 7009 cells overproducing gp2-like proteins encoded by *Pseudomonas* phages (data not shown). The results suggest that T7 gp2 and gp2-like proteins encoded by *Pseudomonas* phages have a common binding site on *E. coli* RNAP, which is destroyed by deletion or charge-altering substitutions in the β' jaw.

Pseudomonas phage proteins inhibit transcription in vitro

The results of *in vivo* analyses are consistent with bioinformatic predictions. However, because the growth defect of T7 2^{am} can be complemented by any host RNAP inhibitor, for example, by the addition of rifampicin at the end of the early transcription stage (Ontell and Nakada, 1980; Mooney et al., 1980), the results do not exclude that some of *Pseudomonas* phage proteins inhibit host RNAP by a mechanism that is distinct from T7 gp2 inhibition mechanism. To address this possibility, phage proteins were purified and their

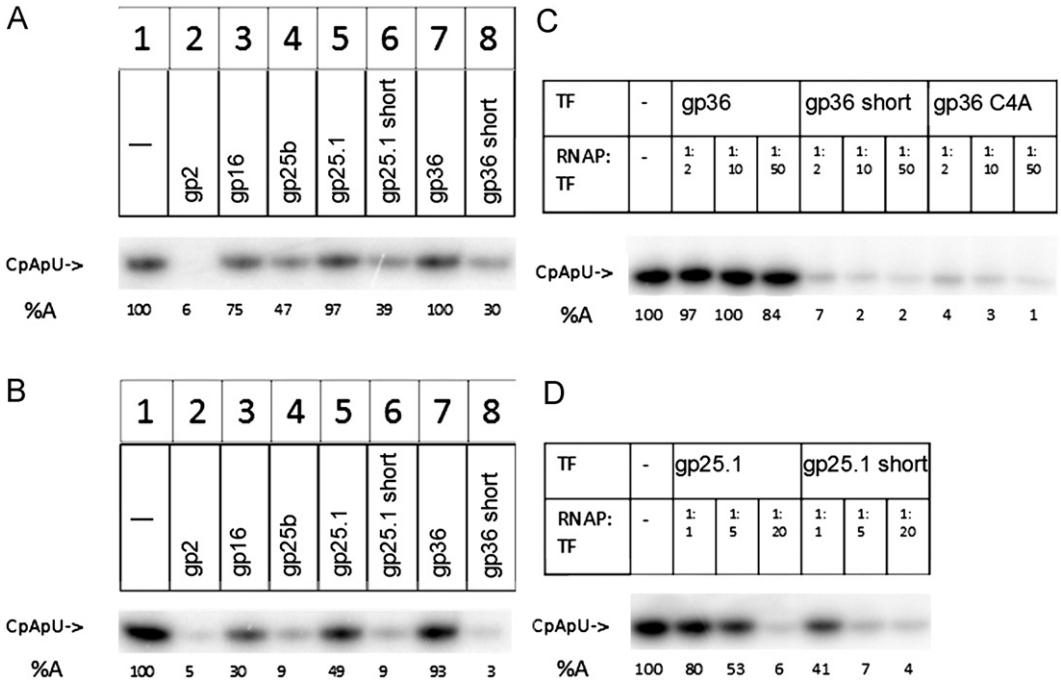

Fig. 3. Transcription inhibition by T7 gp2 and *Pseudomonas* phage proteins. *E. coli* (A) or *P. aeruginosa* (B) RNAPs supplemented with *E. coli* σ^{70} subunit were preincubated with 5xfold molar excess of indicated phage proteins followed by the addition of T7 A1 promoter-containing DNA fragments and substrates for abortive transcription initiation reaction (CpA and α-^{32}P-UTP). Reaction products were revealed after denaturing gel-electrophoresis. The residual activity (amount of the CpApU product synthesized in the presence of phage protein compared to the amount of product synthesized by RNAP alone) is presented below (in %). In panels C and D increasing amounts of indicated phage proteins were added to *P. aeruginosa* RNAP and then analyzed as described above.

ability to inhibit RNAPs purified from *P. aeruginosa* PAO1, and wild-type *E. coli* RNAP was investigated. To ensure that affinity tags used for protein purification do not affect protein function, the tags were removed by treating recombinant purified proteins with thrombine, a specific protease whose cleavage site is located at the boundary between the affinity tag and phage protein sequence. All phage proteins were present in a 5-fold excess over RNAPs. None of phage proteins tested had any effect when added to preformed wild-type *E. coli* or *P. aeruginosa* open promoter complexes (data not shown). The results obtained when phage proteins were combined with RNAP prior to the addition of DNA and transcription substrates are shown in Fig. 3. The data can be summarized as follows. First, T7 gp2 efficiently inhibited transcription by both *E. coli* and *P. aeruginosa* RNAPs (Fig. 3AB, compare lanes 1 and 2). Gp25b, and the short versions of gp25.1 and gp36 were also efficient (less than 10% residual activity) inhibitors of transcription by *P. aeruginosa* RNAP (Fig. 3B, lanes 4, 6, and 8, correspondingly). These proteins appeared to be less efficient inhibitors of the *E. coli* enzyme ($>30\%$ of residual activity, Fig. 3A) in line with earlier observations with the gp2 homolog from phage gh1, which suggested that the interaction with cognate *Pseudomonas* RNAP is stronger than with non-cognate enzyme from *E. coli* (Nechaev et al., 2003). Under the conditions used, the long versions of gp25.1 and gp36 had no effect on *E. coli* RNAP transcription (Fig. 3A, lanes 5 and 7, correspondingly) but partially inhibited the *Pseudomonas* enzyme. Somewhat unexpectedly, phi15 gp16, which is the most closely related to T7gp2, was a relatively poor *in vitro* inhibitor of both *E. coli* and *P. aeruginosa* enzymes (Fig. 3AB, lane 3).

Increasing the concentration of full-sized gp36 up to a 50-excess over RNAP did not lead to transcription inhibition (Fig. 3C). In contrast, when higher concentrations of full-sized gp25.1 were used, complete inhibition of transcription by *P. aeruginosa* RNAP was achieved (Fig. 3D). A shortened version of gp36, as well as the C4A mutant, were highly active in transcription inhibition (Fig. 3C), in agreement with *in vivo* data.

Analysis of pseudomonas phage proteins binding to RNAP

To determine whether *Pseudomonas* phage proteins share the RNAP binding site with T7 gp2, terminally ^{32}P-labeled version of LKD16 gp25b protein was prepared by phosphorylation of the N-terminal heart muscle kinase (HMK) tag. During native PAGE analysis, radioactively labeled LKD16 gp25b interacted with *P. aeruginosa* RNAP as evidenced by formation of a low-mobility radioactive band in the presence of RNAP and the absence of radioactive band corresponding to free LKD16 gp25b (Fig. 4, compare lanes 1 and 2). When a 5-fold excess of unlabeled T7 gp2, phi15 gp16, or LKD16 gp25b was added to reactions containing RNAP and radioactive gp25b, the intensity of the shifted band decreased, and free radioactive gp25b band reappeared (lanes 3, 4, and 5, correspondingly). The results thus prove that the binding of LKD16 gp25b and T7 gp2/phi15 gp16 to RNAP is mutually exclusive. Under the conditions of the experiment, full-sized versions of gp25.1 or gp36 were unable to compete with radioactive LKD16 gp25b (lanes 6 and 8, correspondingly), in agreement with *in vitro* transcription results shown in Fig. 3A. However, the shorter versions of both proteins competed as efficiently as LKD16 gp25b, T7 gp2 or phi15 gp16 (lanes 7 and 9). We therefore conclude that the *Pseudomonas* phage proteins and T7 gp2 share a common binding site on the RNAP.

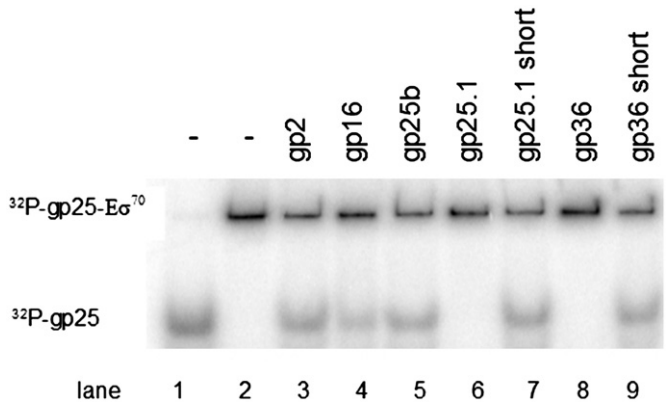

Fig. 4. Binding of phage proteins to *P. aeruginosa* RNAP.^{32} P-labeled gp25b alone (lane 1) or with *P. aeruginosa* RNAP (lane 2) was separated by native gel electrophoresis. In lanes 3–9, reactions contained indicated unlabeled phage proteins present in 5-fold excess over ^{32} P-labeled gp25b.

Discussion

In this communication, we show that several previously uncharacterized gene products of ϕKMV-related *P. aeruginosa* podophages of the T7 supergroup are functional equivalents of bacteriophage T7 gp2 inhibitor of host RNAP. *In vivo* assays reveal that some proteins from ϕKMV-related phages complement the growth defect of T7 phage carrying an amber mutation in gene *2*, provided that the gp2 binding site—the downstream jaw of *E. coli* RNAP–is intact. *In vitro*, some proteins from ϕKMV-related phages inhibit transcription by *P. aeruginosa* and *E. coli* RNAPs and compete with T7 gp2 for the binding to RNAP. Preformed open promoter complexes are resistant to inhibition by both T7 gp2 and ϕKMV-related phage proteins. Overall, the data strongly suggest that transcription inhibitors encoded by ϕKMV-related phage proteins and T7 gp2 have a common transcription inhibition mechanism and act through the same RNAP binding site.

Though our data indicate that host RNAP inhibitors encoded by ϕKMV-related phages and T7 gp2 are also similar by sequence, the question about their homology (origin from a common ancestor) remains open. Secondary structure predictions for gp2 and its close relatives from the T7 subgroup are in very good agreement with T7 gp2 structure determined by NMR (Supplementary Fig. 1). In contrast, transcription inhibitors encoded by ϕKMV-related phages have predicted secondary structures that are radically different from the T7 gp2 secondary structure (Supplementary Figure 1). Cases of homologous proteins assuming different structures have been described, including transcription factor NusG and its paralog RfaH that also interact with RNAP (Belogurov et al., 2007; Bryan and Orban, 2010). Thus, structure determination of one of the ϕKMV subgroup proteins is of considerable interest and is currently underway in our laboratory.

At least two *Pseudomonas* phage proteins, gp25.1 from LUZ19 and gp36 from LKA1, clearly differ from T7 gp2. These proteins are either much longer than T7 gp2 (gp25.1) or contain an N-terminal extension which might form a Zn^{2+} binding site. The «extra» sequences in both proteins appear to interfere with the RNAP inhibition by the C-terminal domain homologous to T7 gp2. In the case of gp36, substitution of cysteine residues that could chelate Zn^{2+} for alanines restores the RNAP inhibitory activity. It is attractive to speculate that N-terminal domains of gp36 (and, possibly, gp25.1) act as receiver modules that allow these proteins to bind to and inhibit the host RNAP at a precise time during post-infection. The nature of the environmental cue(s) that leads to this hypothetical activation of RNAP inhibition by longer proteins encoded by some ϕKMV-related phages remains to be determined. While the N-terminal extension of gp25.1 appears to simply decrease the efficiency of gp2-like domain interaction with (and inhibition of) RNAP, a much shorter N-terminal extension of gp36 completely prevents its inhibitory activity *in vitro* and in a heterologous *E. coli* host *in vivo*. Removing

this extension or substituting one of the four cysteine residues makes gp36 a highly efficient inhibitor of *E. coli* or *P. aeruginosa* RNAP transcription *in vitro*, yet the mutant proteins still only partially complement the growth defect of T7 2^{am}.

The essential function of T7 gp2, and, presumably, of homologs encoded by T7 subgroup phages, is to prevent transcription of phage middle and late genes by host RNAP, which interferes with the viral RNAP transcription. The role of host RNAP inhibitors encoded by ϕKMV-related phages infecting *P. aeruginosa* must be different, as follows from the central position of the viral RNAP gene in these phages genomes, between viral replication (corresponding to middle T7 genes) and virion (corresponding to T7 late genes) gene clusters. If gp2-like proteins encoded by ϕKMV-related phages act as switch factors between host and viral RNAP, they should control a switch from early and middle genes transcription (presumably performed by host RNAP) to late gene transcription performed by viral RNAP.

Available data indicate that among the phages encoding their own RNAP, members of the T7 subgroup, the Xp10-like phages of *Xanthomonas* (Nechaev et al., 2002), and now the ϕKMV-related phages of *P. aeruginosa* encode inhibitors of host RNAP. RNAP-encoding phages such as the *Salmonella* phage SP6, a podovirus, and *E. coli* phage phiEcoM-GJ1 (Jamalludeen et al., 2008), a myovirus, are not known to encode host RNAP inhibitors. We hypothesize that these and other phages encoding their own RNAP encode host transcription inhibitors and could be thus a source of new factors affecting host transcriptional apparatus.

Materials and methods

Affinity purification of bacterial RNAP

A 300 ml culture of *P. aeruginosa* PAO1 cells carrying genomic *rpoC*::Protein A fusion was infected at 37 °C with phage at multiplicity of infection of 10. The infection was terminated 10 min later by rapidly cooling infected cultures. Cells were harvested by centrifugation, washed once with ice-cold 10% (v/v) glycerol, and dissolved in 10 ml Protein A buffer [10 mm Tris.HCl pH 8, 150 mm NaCl, 0.1% (v/v) NP-40] supplemented with 100 µl Protease Inhibitor Mix (Amersham Biosciences), 10 mm Benzonase (Novagen), 10 mg lysozyme (Sigma) and 800 µl 10x BugBuster (Novagen). This mixture was incubated at room temperature for 15 min with gentle agitation. The soluble fraction was isolated by two successive 30-min centrifugation steps at 27,200x g at 4 °C. Subsequently, this fraction was incubated with 200 µl pre-washed IgG SepharoseTM 6 Fast Flow beads (GE Healthcare) with gentle agitation for 2 h at 4 °C. The beads were collected in Bio-Rad Poly-Prep ® Chromatography column and washed three times with Protein A buffer. The beads were incubated in ProTEV Protease (Promega) in the supplied buffer, and transferred to a smaller HandeeTM Spin Column (Pierce) for overnight incubation at 4 °C. The beads were washed twice with Protein A buffer, and all eluates were pooled. The eluted proteins were re-dissolved in 6 M urea/2 M thiorea/10 mm HEPES pH 8.0. Disulfide bounds were reduced by 1 mm dithiothreitol, alkylated with 5.5 mm iodoacetamide, and proteins were digested overnight at 37 °C using modified sequencing grade trypsin (1:50 w/w) after 4-fold diluting the sample with 50 mm NH_4HCO_3. Digestion was halted by the addition of trifluoroacetic acid (TFA) to a final concentration of 1%. Peptides were purified using reversed-phase Sep-Pak C18 peptide purification cartridges, and eluted in 50% acetonitrile/0.1%TFA. The eluates were separated over 130 min on an analytical column (Biosphere C18; NanoSeparations) using a linear gradient from 5 to 60% (v/v) ACN in water containing 100 mm acetic acid. Material eluting from the column was introduced by a nanoelectrospray device and sprayed from a gold-coated fused silica emitter (NanoSeparations). The MS was operated in a data-dependent acquisition mode to automatically switch between MS (m/z 300–1500) and MS/MS acquisition on the three most intense precursor ions, controlled by Xcalibur 1.3 software. MS/MS data were analyzed using Sequest (Thermofinningan) against a local stop-to-stop database of all possible phage proteins allowing 1 Da fragment tolerance. Peptide hits were considered significant at minimal cross-correlation values of 1.8, 2.5 and 3.5 for single, double and triple charged peptide ions, respectively.

Sequence analysis

PSI-BLAST (Altschul et al., 1997) was used to search for gp2 homologs in NCBI NR database or a subset containing only sequences from the *Caudovirales*. The search against sequences from *Caudovirales* was adjusted as follows: expect threshold was set to 100; inclusion threshold 0.001; gap cost parameter existence 9/extension 2; word size 2; no composition based statistics and low complexity filtering were applied. Multiple alignment of the sequences detected by PSI-BLAST was constructed using the Muscle program (Edgar, 2004) with default parameters. Secondary structure was predicted using Jpred (Cole et al., 2008) and PSIPRED (Buchan et al., 2010) programs.

Bacterial strains, phage, and growth conditions

The wild-type bacteriophage $T7^+$ and T7 2^{am} carrying and an amber mutation at gene *2* codon 16 were the same as used in the previous work (Savalia et al., 2010). *E. coli* K-12 strains MG1655 (F- lambda- *ilvG- rfb*-50 *rph*-1) and a Su+ strain IJ511 (Δ*lacX*74 *supE*44 *galK*2 *galT22 mcrA rfbD*1 *mcrB*1 *hsdS*3) were used to propagate the phage.

Cells were grown in standard LB media at 37 °C. To prepare T7 lysates a single plaque was added to 100 ml LB of IJ511 culture grown at 37 °C to mid-log phase (A_{600} 0.3-0.4) and infection was allowed to proceed for 10 h 37 °C. Phage particles were separated from cell debris by the addition of NaCl to 0.5 M, brief shaking, and centrifugation at 10,000 g at +4 °C for 15 min. The resulting lysate had a titer of 2–5 $\times 10^{10}$ p.f.u./ml and was stored at +4 °C.

Plasmids

A pET33-based plasmid expressing T7 gene 2 was described previously (Cámara et al., 2010). Plasmids expressing *Pseudomonas* phage genes were created by cloning PCR fragments amplified from phage genomic DNA with appropriate primers into the multiple cloning site of a pET19-derived vector plasmid. The pET19 commercial vector was modified by site-specific mutagenesis to incorporate a sequence coding for an in-frame HMK tag between the sequence coding for N-terminal histidine tag and the *Nde*I site used for cloning phage ORFs. Both tags can be removed by treating recombinant protein according to manufacturer (Novagene) recommendations.

Phage growth complementation assays

E. coli K-12 MG1655 was transformed with expression plasmids containing cloned genes coding for gp2-like proteins. Transformants were grown at 37 °C in LB until A_{600} 0.4–0.8. 100 µl of T7 2^{am} phage lysate dilution corresponding to ~200 plaque-forming units on IJ511 strain was added to 100 µl cell culture aliquots. After the addition of 4 ml of melted 0.4% LB the infections were overlaid on LB agar plates. Plates were incubated at 37 °C for 10 h and phage plaques were enumerated.

Proteins

An *E. coli* strain JE1134 encoding a T7 gp2-resistant RNAP with deletion of residues 1149–1190 in the β' jaw domain (Ederth et al., 2002) lysogenized with λ(DE3) phage was used for expression of all phage proteins, since most proteins are toxic when expressed in wild-type DE3 lysogens. Transformants were grown in LB supplemented with 100 μg/ml of ampicillin to A_{600} of 0.3–0.5 and expression of plasmid-borne phage genes was induced by the addition of IPTG to 1 mm. After three hours of additional growth, cells were harvested by centrifugation and resuspended in buffer A (20 mm Tris–HCl (pH 8.0), 0.05 M NaCl, 0.5 mM β-mercaptoethanol, 5% glycerol) with 1 mg/ml lysozyme. After 1-h incubation on ice, cells were disrupted by sonication. All recombinant proteins segregated in inclusion bodies. Inclusion bodies were resuspended in buffer B (20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 8 M urea, 0.5 mM β-mercaptoethanol, 5% glycerol). After 1-h incubation at room temperature with occasional mixing, the solution was filtered through Acrodisc® 0.45 Syringe filter and applied on a 1 ml chelating HiTrap column (GE Healthcare) charged with Ni^{2+}. The column was washed with buffer B containing 50 mM imidazole and bound proteins were eluted with buffer B containing 250 mM imidazole. Fractions containing gp2-like proteins were dialyzed into buffer S (20 mM Tris-Cl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 10 mM $ZnCl_2$ and 20% glycerol) overnight, concentrated on Centricon® Centrifugal Filter Units (Millipore), supplemented with glycerol to the final concentration of 50% and resulting proteins (at least 95% pure) were stored at −20 °C.

All RNAPs used in this work were prepared essentially as described previously (Borukhov and Goldfarb, 1993; Kashlev et al., 1996).

In vitro transcription assays

10 μl standard reactions contained of 100 nM Eσ^{70}, 20 nM promoter DNA, 0.5 mM dinucleotide primer CpA, 0.4 μCi of [α-^{32}P]UTP in buffer R (40 mM Tris–HCl, 40 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 100 μg/ml BSA). Unless stated otherwise, phage proteins and Eσ^{70} were always preincubated before the addition of promoter DNA. Reactions were allowed to proceed for 10 min at 37 °C and were terminated by the addition of an equal volume of denaturing loading buffer. The reaction products were resolved on a denaturing 6 M urea 20% (w/v) polyacrylamide gels and visualized using a PhosphorImager.

Native gel mobility assays

Native mobility shift assays and ^{32}P labeling of gp25b were conducted essentially as described previously by Cámara et al., (2010) for T7 gp2. Binding reactions (10 μl) were set up in buffer R at 37 °C and analyzed on a 6% native polyacrylamide gels. The gel was run for 60 min at 100 V. Proteins or protein complexes were visualized and quantified using a PhosphorImager.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.10.021.

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